IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Soe et al.
Serial No.	:	10/040,394
Filed	:	January 9, 2002
Title	:	IMPROVED METHOD FOR PREPARING FLOUR DOUGHS AND PRODUCTS MADE FROM SUCH DOUGHS USING A GLYCEROL OXIDASE
Examiner	:	Keith D. Hendricks
Group Art Unit	:	1761

745 Fifth Avenue, New York, New York 10151

BY HAND DELIVERY

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents
Washington, D.C. 2023
Dear Sir:

Supplemental to the Information Disclosure Statement filed June 16, 2003, the Examiner's attention is respectfully directed to the documents listed on the enclosed PTO-1449, namely U.S. Patents Nos. 6,143,545 and 6,103,505; and, U.S. Provisional application Serial No. 60/039,791, from which the 545 and 505 patents claim a lineage, and which the undersigned received on October 22, 2003. That is the 791 application was ordered, and just received, with this IDS being filed promptly upon receipt.

This information disclosure is not a representation that the cited documents are considered pertinent, or that the cited documents are indeed prior art.

The cited documents herein, namely the 545 and 505 patents and the 791 application, it is respectfully submitted, do not teach or suggest the instant invention.

For instance, it is respectfully submitted that the cited documents fail to teach or suggest a method of preparing a dough comprising adding to dough ingredients an enzyme that hydrolizes compounds including a triglyceride, a glycolipid and a phospholipid.

It is likewise respectfully submitted that the cited documents fail to teach or suggest a method for preparing bread comprising preparing a dough comprising adding to dough ingredients an enzyme that hydrolizes compounds including a triglyceride, a glycolipid and a phospholipid; and baking the dough.

It is further respectfully submitted that the cited documents fail to teach or suggest a method for preparing bread comprising baking a dough prepared by adding to dough ingredients an enzyme that hydrolizes compounds including a triglyceride, a glycolipid and a phospholipid; and baking the dough

It is even further respectfully submitted that the cited documents fail to teach or suggest a dough from the foregoing methods.

Indeed, the cited documents fail to recognize an enzyme that hydrolizes compounds including a triglyceride, a glycolipid and a phospholipid.

Furthermore, it is understood by the undersigned that Applicants invented (conceived, reduced to practice) the instant invention in a GATT/WTO member country prior to the March 4, 1997 filing date of the 791 application such that the 545 and 505 patents and 791 application are believed to be not available as prior art against the instant invention.

Accordingly, it is believed that the documents cited herein present no new issues requiring any further search or examination.

It is respectfully requested that the Examiner consider and make of record the documents herein cited, and that a copy of Form PTO-1449 be initialed by the Examiner and returned to the undersigned. To the extent that a Petition is required for consideration and making of record the documents cited herewith, this paper is to serve as such.

In this regard, as this Information Disclosure Statement is being filed after payment of issue fee, the Commissioner is authorized to charge any required fees or credit any overpayment in fees for considering and making the herewith cited documents of record and/or for any necessary Petition therefor to Deposit Acct. No. 50-0320.

And also in this regard, it is noted that the Rules and the MPEP do not prohibit the Examiner from considering and making of record documents cited at this stage of the prosecution.

Between the Notice of Allowance and the grant of a U.S. Patent, at some point the application itself is removed from the Examining Art Unit and sent to the Publications Branch (the Office of Patent Publication) of the USPTO for actual printing. 37 C.F.R. § 1.97(i) provides that after the Notice of Allowance, the applicant does not have a right to have documents cited in an IDS considered. Indeed, depending on timing, if the application is in the Publications Branch, the Examiner may have no way of knowing that an IDS had been filed.

On the other hand, if an IDS filed after the Notice of Allowance is received by an Examiner, and he decides (1) to consider each document, (2) initial the IDS to indicate that he has done so and (3) the documents cited are to appear on the front of the patent, there is nothing in 37 C.F.R. § 1.97(i) to prevent him from doing so, as is respectfully requested in this application. Simply, it is respectfully recognized that consideration and making of record of the documents cited in this IDS are within the discretion of the USPTO, e.g., the Examiner, which discretion it is respectfully requested is favorably exercised, as such an exercise of discretion is in the interests of justice. *See also* 37 C.F.R. §§ 1.181-1.183; namely that acceptance of this Supplemental Information Disclosure Statement by the Examiner can be within 37 C.F.R. §§ 1.181-1.183, and this paper can be a Petition under such Rules, as it is respectfully submitted that considering and making of record documents cited in this instance can be within the interests of justice, especially because non-acceptance of this IDS can result in the Applicants requesting continued examination, which can protract prosecution and cause a loss of patent term, e.g., due a delay in issuance due to processing of the RCE.

Furthermore, it is respectfully submitted that if the Examiner kindly considers each of the documents cited herein, that such a consideration and making of record of the documents cited herein is wholly consistent with the defined mission of the USPTO to help applicants get valid and enforceable patents on their Inventions. *See, e.g.,* 1998 USPTO Annual Report by then Commissioner Bruce A. Lehman available on the USPTO website at http://www.uspto.gov/web/offices/com/annual/1998/a98r-2.htm#Topic11, "The mission of the patent business area is to help our customers get patents; its performance goal is to grant patents to inventors for their discoveries."

And if in considering a document in an IDS filed after a Notice of Allowance, an Examiner determined that the document would establish a *prima facie* case of unpatentability of an allowed claim, the Examiner has authority to withdraw the Notice of Allowance, reopen the prosecution of the application and reject the claim on the recently cited document. Indeed, 37 C.F.R. § 1.313(a) provides that "[a]pplications may be withdrawn from issue for further action at the initiative of the" USPTO. 37 C.F.R. § 1.313(b) further provides that the USPTO can withdraw an application from issue after payment of the issue fee due to, *inter alia*, unpatentability. And section 1308 of the MPEP provides that:

> An application may be removed from the Office of Patent Publication, without it being withdrawn from issue under 37 CFR 1.313(b), **to permit the examiner to consider an information disclosure statement** or whether one or more claims are unpatentable. Only if such consideration results in a determination that one or more claims are unpatentable does 37 CFR 1.313(b) authorize the application to be withdrawn from issue. [emphasis added]

Thus, it is respectfully submitted that 37 C.F.R. § 1.97(i), and more generally the Rules of the Commissioner, do not prohibit the Examiner from considering an IDS at this stage of the prosecution, or that may not comply with 37 C.F.R. §§ 1.97 and 1.98, such as an IDS after allowance that may not comply with 37 C.F.R. §§ 1.97 and 1.98 (even though such non-compliance is not herein admitted). Rather, it is respectfully submitted that Rules of the Commissioner such as 37 C.F.R. 1.97(i) are a procedural safeguard that prevent Applicants from expecting or insisting upon consideration of an IDS that may not comply with 37 C.F.R. §§ 1.97 and 1.98. (And again, this paper only a very respectful request that the USPTO, e.g., the Examiner, kindly exercise favorable discretion and consider and make of record the documents herewith cited.)

Therefore, it is respectfully requested under all of the Rules, including 37 C.F.R. §§ 1.181-1.183 that the documents cited herein be considered and made of record. The Examiner is respectfully invited to contact the undersigned by telephone should there be any questions.

Respectfully submitted,
FROMMER LAWRENCE & HAUG LLP
Attorneys for Applicants

By Thomas J. Kowalski

Thomas J. Kowalski, Reg. No. 32,147
Tel (212) 588-0800, Fax (212) 588-0500

60/039791

Class | Subclass

TEAM 4

PROVISIONAL APPLICATION NUMBER
60/039791

SERIAL NUMBER	FILING DATE	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
60/039,791 PROVISIONAL	03/04/97				

APPLICANTS

IB GROTH CLAUSEN, HILLEROD, DENMARK; SHAMKANT ANANT PATKAR, COPENHAGEN, DENMARK; TORBEN HALKIER, FREDERIKSBERG, DENMARK; KIM CLAUSEN, TOLLOSE, DENMARK; MARTIN BARFOED, RALEIGH, NC.

CONTINUING DATA********
VERIFIED

FOREIGN/PCT APPLICATIONS********
VERIFIED

FOREIGN FILING LICENSE GRANTED 04/15/97

Foreign priority claimed / 35 USC 119 conditions met / Verified and Acknowledged (Examiner's initials)	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
☐ yes ☐ no / ☐ yes ☐ no	→	DKX	0			$150.01	4798.033

ADDRESS

STEVE T ZELSON
NOVO NORDISK OF NORTH AMERICA
405 LAXINGTON AVENUE SUITE 6400
NEW YORK NY 10174-6001

TITLE

ENZYME OBTAINED FROM A FILAMENTOUS FUNGUS WHICH HAS PHOSPHOLIPASE A AND/OR B ACTICITY

U.S. DEPT. OF COMM./ PAT. & TM—PTO-436L (Rev.12-94)

Form PTO-1625
(Rev. 5/95)

SCAN ☐ C.G. ☐
QC ☐

(FACE) TE

PATENT APPLICATION SERIAL NO. 60/039791

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

PTO-1556
(5/87)

BAR CODE LABEL

U.S. PATENT APPLICATION

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT
60/039,791 PROVISIONAL	03/04/97		

APPLICANT

IB GROTH CLAUSEN, HILLEROD, DENMARK; SHAMKANT ANANT PATKAR, COPENHAGEN, DENMARK; TORBEN HALKIER, FREDERIKSBERG, DENMARK; KIM CLAUSEN, TOLLOSE, DENMARK; MARTIN BARFOED, RALEIGH, NC.

CONTINUING DATA********
VERIFIED

FOREIGN/PCT APPLICATIONS********
VERIFIED

FOREIGN FILING LICENSE GRANTED 04/15/97

STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS	FILING FEE RECEIVED	ATTORNEY DOCKET NO.
DKX	0			$150.00	4798.033

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STEVE T ZELSON
NOVO NORDISK OF NORTH AMERICA
405 LAXINGTON AVENUE SUITE 6400
NEW YORK NY 10174-6001

TITLE

ENZYME OBTAINED FROM A FILAMENTOUS FUNGUS WHICH HAS PHOSPHOLIPASE A AND/OR B ACTICITY

This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application which is identified above.

By authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

Date

Certifying Officer

60/039791

Attorney Docket No.: 4798.033-US PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

EXPRESS MAIL CERTIFICATE

Hon. Commissioner of Patents and Trademarks
Washington, DC 20231

Re: U.S. Provisional Application for
An Enzyme, Obtained From A Filamentous Fungus, Which Is Having Phospholipase A And/Or B Activity
Applicants: Clausen, et al.

Sir:

Express Mail Label No. EM322112965US

Date of Deposit March 4, 1997

I hereby certify that the following attached paper(s) or fee

1. Filing Under 37 C.F.R. §1.53 (in duplicate)
2. Provisional Application

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, DC 20231.

Miriam Kelly
(Name of person mailing paper(s) or fee)

Miriam Kelly
(Signature of person mailing paper(s) or fee)

Mailing Address:

Novo Nordisk of North America, Inc.
405 Lexington Avenue, Suite 6400
New York, NY 10017
(212) 867-0123

o/o

A/PROVI—
60/039791

Attorney Docket No.: 4798.033

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FILING UNDER 37 C.F.R. §1.53 (b) (2)

Commissioner of Patents and Trademarks
Washington, DC 20231

Sir:

This is a request for filing a provisional application under 37 C.F.R. §1.53(b)(2), of the inventors:

Ib Groth Clausen, citizen of Denmark, residing in Hillerød, Denmark;

Shamkant Anant Patkar, citizen of Denmark, residing in Copenhagen, Denmark;

Torben Halkier, citizen of Denmark, residing in Frederiksberg, Denmark;

Kim Clausen, citizen of Denmark, residing in Tølløse, Denmark; and

Martin Barfoed, citizen of Denmark, residing in Raleigh, NC 27604, USA

for application entitled An Enzyme, Obtained From A Filamentous Fungus, Which Has Phospholipase A And/Or B Activity.

The provisional application contains:

[x] 52 pages of specification

Address all future communications to Steve T. Zelson, Esq., Novo Nordisk of North America, Inc., 405 Lexington Avenue, Suite 6400, New York, NY 10174-6401.

Please charge the required fee, estimated to be $150, to Novo Nordisk of North America, Inc., Deposit Account No. 14-1447. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: March 4, 1997

Elias J. Lambiris, Reg. No. 33,728
Novo Nordisk of North America, Inc.
405 Lexington Avenue, Suite 6400
New York, NY 10174-6401
(212) 867-0123

60039791 030497

Attorney Docket No. 4798.033-US

An Enzyme Obtained from a Filamentous Fungus, Which Has Phospholipase A And/or B Activity

FIELD OF INVENTION

The present invention relates to an enzyme with phospholipase activity, a cloned DNA sequence encoding the enzyme with phospholipase activity, a method of producing the enzyme, and the use of said enzyme for a number of industrial applications.

BACKGROUND OF THE INVENTION

Phospholipids, such as lecithin or phosphatidylcholine, consist of glycerol esterified with two fatty acids in an outer (sn-1) and the middle (sn-2) positions and esterified with phosphoric acid in the third position; the phosphoric acid, in turn, may be esterified to an amino-alcohol. Phospholipases are enzymes which participate in the hydrolysis of phospholipids. Several types of phospholipase activity can be distinguished, including phospholipase A_1 (PLA_1) and A_2 (PLA_2) which hydrolyze one fatty acyl group (in the sn-1 and sn-2 position, respectively) to form lysophospholipid; and lysophospholipase (or phospholipase B (PLB)) which can hydrolyze the remaining fatty acyl group in lysophospholipid. This invention relates to a filamentous fungal phospholipase that has the ability to hydrolyze one and/or both fatty acyl groups in a phospholipid (*i.e.* exhibiting PLA and/or PLB activity).

Previously characterized fungal PLA and/or PLB enzymes:

Numerous references describe characterization of fungal phospholipases. In order to make it easier to get an overview of the overall prior art status, the references are ordered in two sections.

Section one deals with references describing the identification of fungal phospholipases presently not believed to be related to the fungal phospholipases of the present invention. Those references are mainly included in order to summarize the state of the art within the field of characterization of fungal phospholipases.

Section two deals with references describing characterization of fungal phospholipases believed to be of some relevance to the fungal phospholipases of the present invention.

References summarizing the state of the art (Section one)

Enzymes with phospholipase A and/or B activity have been reported from various fungal sources, including *Penicillium notatum* (also known as *P. chrysogenum*; N. Kawasaki, J. Biochem., 77, 1233-44, 1975; N. Masuda et al., Eur. J. Biochem., 202, 783-787, 1991), *P. cyclopium* (Process Biochemistry 30(5): 393-401 (1995)), *Saccharomyces cerevisiae* (M. Ichimasa et al., Agric. Biol. Chem., 49 (4), 1083-89, 1985; F. Paultauf et al., J. Biol. Chem., 269, 19725-30, 1994), *Torulaspora delbrueckii* (old name *Saccharomyces rosei*; Y. Kuwabara, Agric. Biol. Chem., 52 (10), 2451-58, 1988; FEMS, Microbiol. Letters, 124, 29-34), *Schizosaccharomyces pombe* (H. Oishi et al., Biosci. Biotech. Biochem., 60 (7), 1087-92, 1996), *Aspergillus niger* (Technical Bulletin, G-zyme™ G999, Enzyme Bio-Systems Ltd.; Process Biochemistry 30(5): 393-401 (1995)) and *Corticium centrifugum* (S. Uehara et al., Agric. Biol. Chem., 43 (3), 517-525, 1979).

Previously characterized fungal phospholipases and references which are related to the present invention. (section two):

EP 575133 A2 describe isolation and characterization of a fungal phoshoslipase A1 obtained from Aspergillus and the use of those for industrial applications.

No sequence information (either DNA or amino acid) is disclosed in the application neither is any strategy or suggestion for cloning any of the Aspergillus phospholipase discussed or indicated in the application.

Tsung-Che et al. (Phytopathological notes 58:1437-38 (1968)) briefly describe characterization of a phospholipase from Fusarium solani.

EP 130,064 describes a lipase obtained from the strain *Fusarium oxysporum*, DSM 2672, for use in detergent compositions. However, EP 130,064 does not describe any enzymes exhibiting any phospholipase activity.

WO 96/13579 describes a lipase obtained from the strain *Fusarium culmorum*, CBS 513.94 including its N-terminal sequence.

However, WO 96/13579 does not describe any enzyme exhibiting phospholipase activity.

A cDNA sequence encoding a lipase from *Fusarium heterosporum* is described (Cloning and nucleotide sequence of cDNA encoding a lipase from *Fusarium heterosporum*, J. Biochem. 116, 536-540, 1994.). This sequence is presently believed to be the most related

DNA sequence compared to a cloned DNA sequence of the invention (See section "Comparison with prior art" (*vide infra*). However, this reference does not describe any enzyme exhibiting phospholipase activity.

A cDNA sequence encoding a phospholipase B from *Penicillum notatum* is described (Eur. J. Biochem 202:783-787, 1991). However this cloned DNA sequence has very limited homology to a DNA sequence of the invention (See section "Comparison with prior art" (*vide infra*).

Industrial application of the phospholipase of the invention:

It is known to use phospholipase in, *e.g.* enzymatic oil degumming (US 5,264,367, Metallgesellschaft, Röhm), treatment of starch hydrolysate (particularly from wheat starch) to improve the filterability (EP 219,269, CPC International) and as an additive to bread dough to improve the elasticity of the bread (US 4,567,046, Kyowa Hakko).

Presently the phospholipase Lecitase® (Novo Nordisk A/S) is used commercially such as for degumming of oils. Lecitase® is a mammalian enzyme obtained from porcine pancreas.

It is well known that it is possible to produce fungal enzymes recombinantly at industrially economically acceptable yields, especially from filamentous fungi.

Consequently, it is the object of this invention to provide an improved phospholipase *e.g.* for use in the processes described above.

Further the present invention disclose a completely new process for degumming of a "crude oil".

Even further it is an object of the present invention to describe processes and methods for recombinant production at industrially acceptable yields of a phospholipase obtained from a filamentous fungi.

SUMMARY OF THE INVENTION

Despite a number of technical difficulties (*vide infra*) the present inventors have been able to clone an enzyme exhibiting phospholipase A activity from a strain of the genus Fusarium, more specifically *Fusarium oxysporum*.

This is believed to be the first time a filamentous fungal phospholipase A has been cloned and consequently for the first time the present invention provides a cloned DNA sequence encoding a filamentous fungal phospholipase A enzyme.

Accordingly, a first aspect of the invention relates to a cloned DNA sequence encoding a polypeptide having phospholipase A activity wherein the DNA sequence is cloned from a filamentous fungus.

A cDNA sequence encoding a phospholipase B from *Penicillium notatum* is described (Eur. J. Biochem 202:783-787, 1991).

However this DNA sequence shows only very limited DNA identity 39% to the DNA sequence of the present invention (SEQ ID NO 1 23-1060), and physiological characteristic such as molecular mass is very different between said PLB from *P. notatum* (66 kDa) and a phospholipase of the invention (29 ± 10 kDa (*vide infra*)).

Further a comparison with prior art nucleotide and amino acid sequences has shown that the DNA sequence and/or the corresponding encoded amino acid sequence of the invention has only little homology to any prior art DNA and/or amino acid sequences (*vide infra*).

Consequently, it is presently believed that the DNA sequence information provided in the present application will be highly valuable in order to *e.g.* clone another related/homologous phospholipase encoding DNA sequence, since a specific hybridization probe and/or PCR primers can now easily be constructed on the basis of said DNA sequence of the invention.

Further it is presently believed that it is possible to clone both a related/homologous phospholipase A and/or phospholipase B encoding DNA sequence based on the sequence information provided in the present application.

Accordingly, in a second aspect the invention relates to a cloned DNA sequence encoding an enzyme exhibiting phospholipase A and/or phospholipase B activity, which DNA sequence is selected from the group comprising of:

(a) the phospholipase A encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Escherichia coli* DSM 11299;

(b) the DNA sequence shown in positions 23-1060 in SEQ ID NO 1 or its complementary strand;

(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 70% homologous with said DNA sequence;

(d) a DNA sequence which hybridizes with the DNA sequence shown in positions 23-1060 in SEQ ID NO 1 at low stringency;

(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequences of (b) or (d), but which codes for a polypeptide having exactly the same amino acid sequences as the polypeptide encoded by these DNA sequences; and

(f) a DNA sequence which is an allelic form or fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

Further amino acid sequence homology studies between a Fusarium oxysporum phospholipase of the invention having the amino acid sequence as shown in positions 1-346 of SEQ ID NO 2 and a related prior art lipase sequence from *Fusarium heterosporum* (See "BACKGROUND" section (*vide supra*))shows that the F. oxysporum phospholipase of the invention has a specific characteristic sequence insertion between amino acid No 307 to 318 of SEQ ID No 2 (see Fig. 1).

Presently it is believed that this specific insertion is very important for the PLA and/or PLB activity of the enzyme of the invention. Further it is presently believed that it is possible to modify, by e.g. substitution, deletion and/or insertions in or at the amino acid(s) of interest, one or two amino acids within this specific insertion, and still keep the for PLA and/or PLB activity important feature of said insertion.

Accordingly, the present invention relates in its third aspect to a filamentous fungal PLA and/or PLB comprising

the amino acid sequence "YRSAESVDKRAT", or

the amino acid sequence defined in (a) where one or two of the amino acid sequences have been modified.

The DNA sequence encoding for said specific amino acid sequence "YRSAESVDKRAT" is "TAC AGA AGC GCC GAG AGC GTC GAC AAG AGG GCC ACC" which is shown in position 941-976 in SEQ ID No 1.

Accordingly, the present invention relates in its fourth aspect to a cloned DNA sequence encoding an enzyme exhibiting PLA and/or PLB activity, which DNA sequence comprises

(a) the DNA sequence "TAC AGA AGC GCC GAG AGC GTC GAC AAG AGG GCC ACC" (positions 941-976 in SEQ ID NO 1); or

(b) an analogue of the DNA sequence defined in (a) which hybridizes with the DNA sequence shown in positions 941-976 in SEQ ID NO 1 at low stringency.

Further a phospholipase of the invention has been intensively characterized and it has been found that it has no significant lipase activity and has phospholipase activity at very low pH; these properties makes it very suitable for use in oil degumming, as enzymatic and alkaline hydrolysis (saponification) of the oil can both be suppressed. The phospholipase is not membrane bound, making it suitable for commercial production and purification.

Accordingly, in a fifth aspect the invention relates to an isolated polypeptide having phopholipase A activity which is obtained from a strain of the genus *Fusarium* and has

(a) PLA activity in the pH range 3-7, measured at 40°C;

(b) a molecular mass of 29 ± 10 kDa, as determined by SDS-PAGE;

(c) an isoelectric point (pI) in the range 4.5-8; and/or

(d) a thermal denaturation temperature (Td) in the range between 52°C to 65°C, measured at pH 4.

In a still further aspect the invention provides a recombinant expression vector, which enables heterologous recombinant production of an enzyme of the invention. Thereby it is possible to make a highly purified phospholipase composition, characterized in being free from homologous impurities. This is highly advantageous for a number of industrial applications.

Further the present inventors have surprisingly identified a new method for degumming of crude oil. This method is a modification of the prior art described oil degumming methods, which are based on degumming of a water-degummed oil (US 5264367, EP 513709, JP-A 2-153997). A number of especially economical advantages are related to this new method, since it now may be possible to enzymatically degumming a crude oil, without having to include a preliminary water-degumming step ("wet refining to remove mucilage": US 5264367).

Accordingly, a further aspect of the invention relates to a method for reducing the content of phosphorus containing components in a crude edible oil, which method comprises contacting said oil at a pH from 3-6 with an aqueous solution of a phospholipase which is emulsified in the oil until the phosphorus content of the oil is reduced to less than 11 ppm, and then separating the aqueous phase from the treated oil.

Finally the invention relates to an isolated substantially pure biological culture of the *Escherichia coli* strain DSM No. 11299 harbouring a phospholipase encoding DNA sequence (the phospholipase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present

in *Escherichia coli* DSM 11299) obtained from a strain of the filamentous fungus *Fusarium oxysporum*, or any mutant of said *E.coli* strain having retained the phospholipase encoding capability; and to an isolated substantially pure biological culture of the filamentous fungus *Fusarium oxysporum* DSM No. 2672, from which the DNA sequence presented as SEQ ID No. 1 has been derived.

Comparison with prior art

A homology search with the phospholipase of the invention against nucleotide and protein databases was performed. The homology search showed that the most related known sequence was a lipase from *Fusarium heterosporum* (An amino acid alignment is illustrated in figure 1).

The DNA sequence of the invention (SEQ ID NO 1 23-1060) encoding the phospholipase shows only 62% DNA identity to the known lipase sequence from *Fusarium heterosporum* (Genbank database reference S77816), and the corresponding amino acid sequence of the phospholipase of the invention (SEQ ID NO 2) shows only 60% identity to a deduced amino acid sequence based on the known DNA sequence above (see figure 1).

This shows that the DNA and/or the amino acid sequence of a phospholipase of the invention indeed is distant from any known DNA and/or the amino acid sequence(s).

A cDNA sequence encoding a phospholipase B from *Penicillum notatum* is described (Eur. J. Biochem 202:783-787, 1991). However this DNA sequence (Genbank database reference X60348) shows only very limited DNA identity 39% to the DNA sequence of the present invention (SEQ ID NO 1 23-1060), and the corresponding amino acid sequence of the phospholipase of the invention (SEQ ID NO 2) shows only 20% identity to a deduced amino acid sequence based on the known PLB DNA sequence above.

The calculation of homology was done as described later in this specification.

DEFINITIONS

Prior to discussing this invention in further detail, the following terms will first be defined.

"A cloned DNA sequence": The term "A cloned DNA sequence", refers to a DNA sequence cloned in accordance with standard cloning procedures used in genetic engineering to relocate a segment of DNA from its natural location to a different site where it will be

reproduced. The cloning process involves excision and isolation of the desired DNA segment, insertion of the piece of DNA into the vector molecule and incorporation of the recombinant vector into a cell where multiple copies or clones of the DNA segment will be replicated.

The "cloned DNA sequence" of the invention may alternatively be termed "DNA construct" or "isolated DNA sequence".

"Obtained from": For the purpose of the present invention the term "obtained from" as used herein in connection with a specific microbial source, means that the enzyme is produced by the specific source, or by a cell in which a gene from the source have been inserted.

"An isolated polypeptide": As defined herein the term, "an isolated polypeptide" or "isolated phospholipase", as used about the phospholipase of the invention, is a phospholipase or phospholipase part which is essentially free of other non-phospholipase polypeptides, e.g., at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by SDS-PAGE.

When the isolated polypeptide is at least 60% pure the term "A highly isolated polypeptide" may be used.

The term "isolated polypeptide" may alternatively be termed "purified polypeptide".

"Homologous impurities": As used herein the term "homologous impurities" means any impurity (e.g. another polypeptide than the enzyme of the invention) which originate from the homologous cell where the enzyme of the invention is originally obtained from. In the present invention the homologous cell may *e.g.* be a strain of *Fusarium oxysporum.*

"Phospholipase encoding part": As used herein the term "phospholipase encoding part" used in connection with a DNA sequence means the region of the DNA sequence which corresponds to the region which is translated into a polypeptide sequence. In the DNA sequence shown in SEQ ID NO 1 it is the region between the first "ATG" start codon ("AUG" codon in mRNA) and the following stop codon ("TAA", "TAG" or "TGA").

The translated polypeptide may further, in addition to the mature sequence exhibiting phospholipase activity, comprise an N-terminal signal and/or a pro-peptide sequence. The signal sequence generally guides the secretion of the polypeptide and the pro-peptide generally guides the folding of the polypeptide. For further information see Egnell, P. et al.

Molecular Microbiol. 6(9):1115-19 (1992) or Stryer, L., "Biochemistry" W.H., Freeman and Company/New York, ISBN 0-7167-1920-7.

"Modification(s) of a DNA and/or amino acid sequence" The term "modification(s)" used in connection with modification(s) of a DNA and/or amino acid sequence as discussed herein is defined to include chemical modification as well as genetic manipulation(s). The modification(s) can be by substitution, deletion and/or insertions in or at the amino acid(s) of interest.

"Phospholipase A" The term "Phospholipase A" used herein in connection with an enzyme of the invention is intended to cover an enzyme with Phospholipase A1 and/or Phospholipase A2 activity.

Phospholipase A1 is defined according to standard enzyme EC-classification as EC 3.1.1.32.

Official Name: Phospholipase A1 (PLA1).

Reaction catalyzed:

phosphatidylcholine + H(2)O < >

2-acylglycerophosphocholine + a fatty acid anion

Comment(s)

has a much broader specificity than ec 3.1.1.4.

Phospholipase A2 is defined according to standard enzyme EC-classification as EC 3.1.1.4

Official Name: phospholipase A2 (PLA2).

Alternative Name(s):phosphatidylcholine 2-acylhydrolase.

lecithinase a; phosphatidase; or phosphatidolipase.

Reaction catalysed:

phosphatidylcholine + H(2)O < >

1-acylglycerophosphocholine + a fatty acid anion

comment(s): also acts on phosphatidylethanolamine, choline plasmalogen and phosphatides, removing the fatty acid attached to the 2-position.

"Phospholipase B": Phospholipase B is defined according to standard enzyme EC-classification as EC 3.1.1.5.

Official Name:lysophospholipase.

Alternative Name(s):lecithinase b; lysolecithinase;

phospholipase b; or plb.

Reaction catalysed:

2-lysophosphatidylcholine + h(2)o < > glycerophosphocholine + a fatty acid anion

"Phospholipase activity" The term "phospholipase activity" or "having/exhibiting phospholipase activity" as used herein in connection with an enzyme of the invention is intended to specify an enzyme having at least the amount of phospholipase activity (be it PLA or PLB) experimentally defined below.

Accordingly, an enzyme exhibiting phospholipase activity is herein defined as an enzyme which in the "monolayer phospholipase assay" shown in Example 6 herein (*vide infra*) is having a phospholipase activity of at least 0.25 nmol/min, Enzyme dose: 60 μg; more preferably at least 0.40 nmol/min, Enzyme dose: 60 μg; more preferably at least 0.75 nmol/min, Enzyme dose: 60 μg; more preferably at least 1.0 nmol/min, Enzyme dose: 60 μg; more preferably at least 1.25 nmol/min, Enzyme dose: 60 μg; and even more preferably at least 1.5 nmol/min, Enzyme dose: 60 μg.

It is presently believed that only an enzyme having such a significant phospholipase activity is of industrial importance, for instance for use in degumming (US 5.264.367).

"A lipase with phospholipase side activity": The term "lipase with phospholipase side activity" is accordingly defined as a lipase with a phospholipase side activity wherein the phospholipase side activity in the "monolayer phospholipase assay" shown in Example 6 is less than the above mentioned figures specifying phospholipase activity.

A number of lipases is having such phospholipase side activity. In working example 6 herein (*vide infra*) is shown some of such lipases having phospholipase side activity.

"A crude oil" A crude oil (also called a non-degummed oil) may be a pressed or extracted oil or a mixture thereof from e.g. rapeseed, soybean, sunflower. The phosphatide content in a crude oil may vary from 0.5-3% w/w corresponding to phosphorus contents in the range 200-1200 ppm. Apart from the phosphatides the crude oil also contains small concentrations of carbohydrates, sugar compounds and metal/phosphatide acid complexes of Ca, Mg and Fe.

"A semicrude oil" Any oil which is not a crude oil, but which has a phosphatide content above 250 ppm. Such an oil could e.g. be achieved by subjecting a crude oil to a process similar to the "water degummed oil" process described below.

"A water-degummed oil" A water-degummed oil is typically obtained by mixing 1-3% w/w of hot water into warm (60-90°C) crude oil. Usual treatment periods are 30-60 minutes. The water-degumming step removes the phosphatides and mucilaginous gums that when hydrated become insoluble in the oil. The hydrated phosphatides and gums can be separated from the oil by settling, filtering or centrifuging - centrifuging being the more prevalent practice.

Alternatively the process here termed "A water-degummed oil" may be called "wet refining to remove mucilage" (see US 5264367).

DETAILED DESCRIPTION OF THE INVENTION

Characterization of Phospholipase obtained from *Fusarium oxysporum*.

A phospholipase of the invention obtained from *Fusarium oxysporum* has been intensively characterized.

Accordingly, an aspect of the invention is preferably an isolated Phosholipase A which is obtained from a strain of the genus *Fusarium* and has phospholipase A activity in the pH range between pH 3-7 measured at 40°C, more preferably having phospholipase A activity in the pH range 3.5-6 measured at 40°C, and even more preferably having phospholipase A activity in the pH range 4-5 measured at 40°C.

The phospholipase A activity was determined with Soybean Lecithin as substrate as described in a working example herein (NEFA test bases assay).

In a further embodiment of the invention, an isolated Phosholipase A which is obtained from a strain of the genus *Fusarium* is preferably one which has a molecular mass of 29 ± 10 kDa, more preferably a molecular mass of 29 ± 5 kDa, even more preferably a molecular mass of 29 ± 3 kDa, and most preferably a molecular mass of 29 ± 2 kDa.

The molecular mass is measured by SDS-PAGE electrophoresis as further described in the "Materials and Methods" section (*vide infra*).

In a further embodiment of the invention, an isolated Phosholipase A which is obtained from a strain of the genus *Fusarium* is preferably one which has an isoelectric point (pI) in the range 4.5-8, more preferably an isoelectric point (pI) in the range 5-7.5, and even more preferably an isoelectric point (pI) in the range 5.5-7.5.

The Isoelectric point (pI) was determined by using Ampholine PAGE plates from Pharmacia. See working example herein for further details (*vide infra*).

In a further embodiment of the invention, an isolated Phospholipase A which is obtained from a strain of the genus *Fusarium* is preferably one which has a thermal denaturation temperature (Td) in the range between 50°C and 65°C, measured at pH 4; more preferably a thermal denaturation temperature (Td) in the range between 52°C and 63°C, measured at pH 4; and even more preferably a thermal denaturation temperature (Td) in the range between 54°C and 60°C, measured at pH 4.

The thermal denaturation temperature (Td) is investigated by means of DSC (Differential Scanning Calorimetry). The thermal denaturation temperature, Td, was taken as the top of denaturation peak in thermograms (Cp vs. T) obtained after heating of enzyme solutions at a constant programmed heating rate. See working example herein for further details (*vide infra*).

The N-terminal amino acid sequence of an isolated Phospholipase A which is obtained from a strain of the genus *Fusarium*, more specifically *Fusarium oxysporum*, has been determined. See working example herein for further details (*vide infra*).

Further this enzyme has been cloned (*vide infra*) and the deduced amino acid sequence of the phospholipase is shown in SEQ ID No 2.

Further it is presently believed it is possible to clone both a related/homologous phospholipase A and/or phospholipase B encoding DNA sequence based on the sequence information (both DNA and/or amino acid) provided in the present application.

Accordingly, an further aspect of the invention is an isolated enzyme exhibiting phospholipase A and/or B activity selected from the group comprising of:

(a) a polypeptide encoded by the phospholipase A and/or B enzyme encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in Escherichia coli DSM 11299;

(b) a polypeptide having an amino acid sequence as shown in positions 31-346 of SEQ ID NO 2;

(c) an analogue of the polypeptide defined in (a) or (b) which is at least 70 % homologous with said polypeptide; and

(d) an allelic form or fragment of (a), (b) or (c).

Preferably a phospholipase, having the characteristic shown above, of the invention is obtained from a strain of *Fusarium oxysporum*. However, without being limited to any theory it is at present contemplated that a phospholipase of the invention can also be obtained

from another filamentous fungus. Examples of those are given in the section "Microbial sources" (*vide infra*).

Cloned DNA sequence

Despite a number of technical difficulties (See section "Protocol for cloning a filamentous fungal phospholipase", *vide infra*) the present inventors have been able to clone a phospholipase, exhibiting PLA activity, from a strain of the genus *Fusarium*, more specifically *Fusarium oxysporum*.

Further it is presently believed that it is possible to clone both a related phospholipase A and/or phospholipase B encoding DNA sequence based on the sequence information provided in the present application.

Accordingly, an aspect of the invention relates to a cloned DNA sequence encoding an enzyme exhibiting phospholipase A and/or phospholipase B activity, which DNA sequence is selected from the group comprising of:

(a) the phospholipase A encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Escherichia coli* DSM 11299;

(b) the DNA sequence shown in positions 23-1060 in SEQ ID NO 1 or its complementary strand;

(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 70% homologous with said DNA sequence;

(d) a DNA sequence which hybridizes with the DNA sequence shown in positions 23-1060 in SEQ ID NO 1 at low stringency;

(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequences of (b) or (d), but which codes for a polypeptide having exactly the same amino acid sequences as the polypeptide encoded by these DNA sequences; and

(f) a DNA sequence which is a allelic form or fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

In this specification, whenever reference is made to the phospholipase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in DSM 11299 such reference is also intended to include the phospholipase encoding part of the DNA sequence presented in SEQ ID NO 1.

Accordingly, the terms "the phospholipase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in DSM 11299" and "the phospholipase encoding part of the DNA sequence presented in SEQ ID NO 1" may be used interchangeably.

The DNA sequence may be of genomic, cDNA, or synthetic origin or any combination thereof.

The present invention also encompasses a cloned DNA sequence which encodes an enzyme exhibiting phospholipase A and/or phospholipase B activity having the amino acid sequence set forth as the mature part of SEQ ID NO 2, which differ from SEQ ID NO 1 by virtue of the degeneracy of the genetic code.

The DNA sequence shown in SEQ ID NO 1 and/or an analogue DNA sequence of the invention may be cloned from a strain of the filamentous fungus *Fusarium oxysporum* producing the enzyme with phospholipase activity, or another or related organism as further described below (See section "Microbial sources").

Alternatively, the analogous sequence may be constructed on the basis of the DNA sequence presented as the phospholipase encoding part of SEQ ID No. 1, *e.g.* be a subsequence thereof, and/or be constructed by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the phospholipase encoded by the DNA sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence (*i.e.* a variant of the phospholipase of the invention).

When carrying out nucleotide substitutions, amino acid changes are preferably of a minor nature, *i.e.* conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification, such as a poly-histidine tract; an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids, such as arginine, lysine, histidine; acidic amino acids, such as glutamic acid and aspartic acid; polar amino acids, such as glutamine and asparagine; hydrophobic amino acids, such as leucine, isoleucine, valine; aromatic amino acids, such as phenylalanine, tryptophan, tyrosine; and small amino acids, such as glycine, alanine, serine, threonine, methionine. For

a general description of nucleotide substitution, see *e.g.* Ford et al., (1991), Protein Expression and Purification 2, 95-107.

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the cloned DNA sequence of the invention, and therefore preferably not subject to substitution may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (cf. *e.g.* Cunningham and Wells, (1989), Science 244, 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (*i.e.* phospholipase) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photo affinity labelling (cf. *e.g.* de Vos et al., (1992), Science 255, 306-312; Smith et al., (1992), J. Mol. Biol. 224, 899-904; Wlodaver et al., (1992), FEBS Lett. 309, 59-64).

Polypeptides of the present invention also include fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleic acid sequence (or a portion thereof) encoding another polypeptide to a nucleic acid sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include, ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

The DNA sequence of the invention can be cloned from the strain *Escherichia coli* DSM No. 11299 using standard cloning techniques *e.g.* as described by Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

The DNA sequence may also be cloned from an organism producing said enzyme, e.g. by purifying the enzyme, amino acid sequencing, and preparing a suitable probe or PCR primer based on this amino acid sequence.

The DNA sequence of the invention can also be cloned by any general method involving

a. cloning, in suitable vectors, a cDNA library from any organism expected to produce the phospholipase of interest,

b. transforming suitable yeast host cells with said vectors,

c. culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the cDNA library,

d. screening for positive clones by determining any phospholipase activity of the enzyme produced by such clones, and

e. isolating the enzyme encoding DNA from such clones.

A general isolation method has been disclosed in WO 93/11249 and WO 94/14953, the contents of which are hereby incorporated by reference. A more detailed description of the screening method is given in a working example herein (*vide infra*).

Alternatively, the DNA encoding a phospholipase of the invention may, in accordance with well-known procedures, conveniently be cloned from a suitable source, such as any of organisms mentioned in the section "Microbial Sources", by use of synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of the phospholipase encoding part of the nucleotide sequences presented as SEQ ID No. 1 or any suitable subsequence thereof, or the basis of the amino acid sequence SEQ ID NO 2.

Protocol for cloning a filamentous fungal phospholipase

A number of technical difficulties have been solved by the present inventors in order to be able to clone a filamentous fungal phospholipase of the invention.

As described herein no prior DNA sequence encoding a filamentous fungal phospholipase A was available. Consequently the present inventors developed a cloning strategy based on the known expression cloning in yeast technique (H. Dalboege et al. Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953).

One of the major problems encountered with this technique was that yeast produces an internal activity giving rise to a phospholipase background on plate assays. The background was found to be highly dependent on the amount of substrate in the assay plates, and the amount of substrate thus had to be carefully titrated to a level where the background was low enough for the assay to be reliable during the expression cloning screening procedure, but high enough for the reaction to take place.

In the Materials and Method section is disclosed a detailed description of the complete expression cloning in yeast protocol, including a plate assay solving the above described problems.

Homology of DNA sequences

The DNA sequence homology referred to above is determined as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity preferably of at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97% with the phospholipase encoding part of the DNA sequence shown in SEQ ID No. 1.

Hybridization

The hybridization referred to above is intended to comprise an analogous DNA sequence which hybridizes to the nucleotide probe corresponding to the phospholipase encoding part of the DNA sequence shown in SEQ ID NO 1, i.e. nucleotides 23-1060, under at least low stringency conditions as described in detail below.

Suitable experimental conditions for determining hybridization at low, medium, or high stringency between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al. 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 μg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing 10 ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/μg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at least 55°C (low stringency), more preferably at least 60°C (medium stringency), still more preferably at least

65°C (medium/high stringency), even more preferably at least 70°C (high stringency), even more preferably at least 75°C (very high stringency).

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

It has been found that it is possible to theoretically predict whether or not two given DNA sequences will hybridize under certain specified conditions.

Accordingly, as an alternative to the above described experimental method the determination whether or not an analogous DNA sequence will hybridize to the nucleotide probe described above, can be based on a theoretical calculation of the Tm (melting temperature) at which two heterologous DNA sequences with known sequences will hybridize under specified conditions (e.g. with respect to cation concentration and temperature).

In order to determine the melting temperature for heterologous DNA sequences (Tm(hetero)) it is necessary first to determine the melting temperature (Tm(homo)) for homologous DNA sequences.

The melting temperature (Tm(homo) between two fully complementary DNA strands (homoduplex formation) may be determined by use of the following formula,

Tm(homo) = 81.5°C + 16.6(log M) + 0.41(%GC) - 0.61 (% form) - 500/L ("Current protocols in Molecular Biology". John Wiley and Sons, 1995), wherein

"M" denotes the molar cation concentration in wash buffer,

"%GC" % Guanine (G) and Cytosine (C) of total number of bases in the DNA sequence,

"% form" % formamid in the wash buffer, and

"L" the length of the DNA sequence.

Using this formula and the experimental wash conditions given above, Tm(homo) for the homoduplex formation of the nucleotide probe corresponding to the DNA sequence shown in SEQ ID NO 1, i.e. nucleotides 23-1060 is:

Tm(homo) = 81.5 + 16.6 (log 0.30) + 0.41(56) - 0.61(0) - (500/1038)

Tm(homo) = 103.5° C.

"M": 2 X SSC corresponds to a cation conc. of 0.3M.

"%GC" The %GC in SEQ ID No 1 pos. 23-1060 is 56%

"% form": There is no formamid in the wash buffer.

"L": The length of SEQ ID No 1 SEQ ID No 1 pos. 23-1060 1038 bp.

The Tm determined by the above formula is the Tm of a homoduplex formation (Tm(homo)) between two fully complementary DNA sequences. In order to adapt the Tm value to that of two heterologous DNA sequences, it is assumed that a 1% difference in nucleotide sequence between the two heterologous sequences equals a 1°C decrease in Tm ("Current protocols in Molecular Biology". John Wiley and Sons, 1995). Therefore, the Tm(hetero) for the heteroduplex formation is found by subtracting the homology % difference between the analogous sequence in question and the nucleotide probe described above from the Tm(homo). The DNA homology percentage to be subtracted is calculated as described herein (*vide supra*).

Homology to amino acid sequences

The polypeptide homology referred to above is determined as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the mature part of a polypeptide encoded by an analogous DNA sequence exhibits a degree of identity preferably of at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, especially at least 97% with the mature part of the amino acid sequence shown in SEQ ID NO 2, *i.e.* position 31-346 in SEQ ID NO 2.

The present invention is also directed to phospholipase variants which have an amino acid sequence which differs by no more than three amino acids, preferably by no more than two amino acids, and more preferably by no more than one amino acid from the mature part of the amino acid sequence set forth in SEQ ID NO 2.

Immunological cross-reactivity

Antibodies to be used in determining immunological cross-reactivity may be prepared by using a purified phospholipase. More specifically, antiserum against the phospholipase of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen et al. in A Manual of Quantitative

Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically p. 27-31). Purified immunoglobulins may be obtained from the antiserum obtained, for example by salt precipitation ($(NH_4)_2$ SO_4), followed by dialysis and ion exchange chromatography, *e.g.* on DEAE-Sephadex. Immunochemical characterization of proteins may be performed either by Outcherlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

Microbial Sources

At the priority date of the present invention, the taxonomy applied below are in accordance with the World Wide web (WWW) NCBI taxonomy browser.

The phospholipase and the corresponding cloned DNA sequence of the invention may be obtained from any filamentous fungal strain.

A preferred phylum is *Ascomycota*, wherein a preferred class is *Pyrenomycetes* comprising the preferred family *Nectriaceae*.

Even more preferably the phospholipase and the corresponding cloned DNA sequence of the invention may be obtained from a strain of the genus *Fusarium*, in particular *Fusarium oxysporum*.

An isolate of a strain of *Fusarium oxysporum* from which a phospholipase of the invention can be obtained from has been deposited according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutche Sammlung von Mikroorganismen und Zellkulturen GmbH., Masheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany, (DSM).

Deposit date : 15 June 1983

Depositor's ref. : NN041759

DSM No. : *Fusarium oxysporum* DSM No. 2672

Further, the expression plasmid pYES 2.0 comprising the full length cDNA sequence encoding the phospholipase of the invention has been transformed into a strain of the *Escherichia coli* which was deposited according to the Budapest Treaty on the International

Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutche Sammlung von Mikroorganismen und Zellkulturen GmbH., Masheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany, (DSM).

Deposit date : 25 November 1996

Depositor's ref. : NN049279

DSM No. : *Escherichia coli* DSM No. 11299

Expression vectors

The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which the vector it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated tôgether with the chromosome(s) into which it has been integrated.

In the expression vector, the DNA sequence encoding the phospholipase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins which are either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the phospholipase, the promoter and the terminator and to insert them into suitable vectors are well known to persons skilled in the art (cf. *e.g.* Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

Examples of suitable promoters for use in filamentous fungus host cells are, *e.g.* the ADH3 promoter (*McKnight et al.*, The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral α-amylase, *Aspergillus niger* acid stable α-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (glaA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase.

Host Cells

The present invention also relates to recombinant host cells, comprising a nucleic acid sequence of the invention, which are advantageously used in the recombinant production of the polypeptides. The term "host cell" encompasses any progeny of a parent cell which is not identical to the parent cell due to mutations that occur during replication.

The cell is preferably transformed with a vector comprising a nucleic acid sequence of the invention followed by integration of the vector into the host chromosome. "Transformation" means introducing a vector comprising a nucleic acid sequence of the present invention into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleic acid sequence is more likely to be stably maintained in the cell. Integration of the vector into the host chromosome may occur by homologous or non-homologous recombination as described above.

In a preferred embodiment, the host cell is a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*). Representative groups of Ascomycota include, *e.g.*, *Neurospora*, *Eupenicillium* (=*Penicillium*), *Emericella* (=*Aspergillus*), *Eurotium* (=*Aspergillus*), and the true yeasts listed above. Examples of Basidiomycota include mushrooms, rusts, and smuts. Representative groups of Chytridiomycota include, *e.g.*, *Allomyces*, *Blastocladiella*, *Coelomomyces*, and aquatic fungi. Representative groups of Oomycota include, *e.g.*, Saprolegniomycetous aquatic fungi (water molds) such as *Achlya*. Examples of mitosporic fungi include *Aspergillus*, *Penicillium*, *Candida*, and *Alternaria*. Representative groups of Zygomycota include, *e.g.*, *Rhizopus* and *Mucor*.

In a preferred embodiment, the fungal host cell is a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are characterized by a vegetative mycelium composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as

Saccharomyces cerevisiae is by budding of a unicellular thallus and carbon catabolism may be fermentative. In a more preferred embodiment, the filamentous fungal host cell is a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, and *Trichoderma* or a teleomorph or synonym thereof. In an even more preferred embodiment, the filamentous fungal host cell is an *Aspergillus* cell. In another even more preferred embodiment, the filamentous fungal host cell is an *Acremonium* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Fusarium* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Humicola* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Mucor* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Myceliophthora* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Neurospora* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Penicillium* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Thielavia* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Tolypocladium* cell. In another even more preferred embodiment, the filamentous fungal host cell is a *Trichoderma* cell. In a most preferred embodiment, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or *Aspergillus oryzae* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Fusarium* cell of the section Discolor (also known as the section Fusarium). In another preferred embodiment, the filamentous fungal parent cell is a *Fusarium* strain of the section Elegans, *e.g.*, *Fusarium oxysporum*. In another most preferred embodiment, the filamentous fungal host cell is a *Humicola insolens* or *Thermomyces lanuginosa* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Rhizomucor miehei* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Myceliophthora thermophilum* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Neurospora crassa* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Penicillium purpurogenum* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Thielavia terrestris* cell. In another most preferred embodiment, the *Trichoderma* cell is a *Trichoderma*

harzianum, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei* or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81:1470-1474. A suitable method of transforming *Fusarium* species is described by Malardier *et al.*, 1989, *Gene* 78:147-156 or in copending US Serial No. 08/269,449. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology*, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153:163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:1920. Mammalian cells may be transformed by direct uptake using the calcium phosphate precipitation method of Graham and Van der Eb (1978, *Virology* 52:546).

Method of producing phospholipase

The present invention provides a method of producing an isolated enzyme according to the invention, wherein a suitable host cell, which has been transformed with a DNA sequence encoding the enzyme, is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

When an expression vector comprising a DNA sequence encoding the enzyme is transformed into a heterologous host cell it is possible to enable heterologous recombinant production of the enzyme of the invention.

Thereby it is possible to make a highly purified phospholipase composition, characterized in being free from homologous impurities.

In the present invention the homologous host cell may be a strain of *Fusarium oxysporum*.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed phospholipase may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or

filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Use of phospholipase

The phospholipase of the invention can be used in any application where it is desired to hydrolyze the fatty acyl group(s) of a phospholipid or lyso-phospholipid, such as lecithin or lyso-lecithin. The phospholipase is preferably used at pH 1.5-5 (particularly 2-4, e.g. 2-3) and at 30-70°C (particularly 40-60°C). If desired, the phospholipase may be inactivated after the reaction by a heat treatment, e.g. at pH 7, 80°C for 1 hour or 90°C for 10 minutes.

As an example, the phospholipase of the invention can be used in the preparation of dough, bread and cakes, e.g. to improve the elasticity of the bread or cake. Thus, the phospholipase can be used in a process for making bread, comprising adding the phospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4,567,046 (Kyowa Hakko), JP-A 60-78529 (QP Corp.), JP-A 62-111629 (QP Corp.), JP-A 63-258528 (QP Corp.) or EP 426211 (Unilever).

The phospholipase of the invention can also be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the phospholipase. This is particularly applicable to a solution or slurry containing a starch hydrolysate, especially a wheat starch hydrolysate since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

Treatment of vegetable oil (e.g. degumming of vegetable oil)

The phospholipase of the invention can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the phospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil.

Prior to the enzymatic treatment, the vegetable oil is preferably pretreated to remove slime (mucilage), e.g. by wet refining. Typically, the oil will contain 50-250 ppm of

phosphorus as phospholipid at the start of the treatment with phospholipase, and the process of the invention can reduce this value to below 5 ppm.

The enzymatic treatment is conducted by dispersing an aqueous solution of the phospholipase, preferably as droplets with an average diameter below 10 μ(micro)m. The amount of water is preferably 0.5-5% by weight in relation to the oil. An emulsifier may optionally be added. Mechanical agitation may be applied to maintain the emulsion.

The enzymatic treatment can be conducted at any pH in the range 1.5-5. It may be preferred to use a pH of 1.5-3 (e.g. 2-3) in order to suppress the alkaline hydrolysis of triglycerides (saponification). The pH may be adjusted by adding citric acid, a citrate buffer or HCl.

A suitable temperature is generally 30-70°C (particularly 40-60°C). The reaction time will typically be 1-12 hours (e.g. 2-6 hours), and a suitable enzyme dosage will usually be 100-5000 IU per liter of oil, particularly 200-2000 IU/l.

The enzymatic treatment may be conducted batchwise, e.g. in a tank with stirring, or it may be continuous, e.g. a series of stirred tank reactors.

The enzymatic treatment is followed by separation of an aqueous phase and an oil phase. This separation may be performed by conventional means, e.g. centrifugation. The aqueous phase will contain phospholipase, and the enzyme may be re-used to improve the process economy.

In other respects, the process can be conducted according to principles known in the art, e.g. in analogy with US 5,264,367 (Metallgesellschaft, Röhm); K. Dahlke & H. Buchold, INFORM, 6 (12), 1284-91 (1995); H. Buchold, Fat Sci. Technol., 95 (8), 300-304 (1993); JP-A 2-153997 (Showa Sangyo); or EP 654,527 (Metallgesellschaft, Röhm).

Degumming of crude edible oil

The present inventors have surprisingly identified a new method for degumming of crude oil. This method is a modification of the prior art described oil degumming methods, which are based on degumming of a water-degummed oil (US 5264367, EP 513709, JP-A 2-153997). A number of especially economical advantages are related to this new method, since it now may be possible to enzymatically degumming a crude oil, without having to include a preliminary water-degumming step ("wet refining to remove mucilage": US 5264367).

Accordingly, a further aspect of the invention relates to a method for reducing the content of phosphorus containing components in a crude edible oil, which method comprises contacting said oil at a pH from 3-6 with an aqueous solution of a phospholipase which is emulsified in the oil until the phosphorus content of the oil is reduced to less than 11 ppm, and then separating the aqueous phase from the treated oil.

Preferably a phospholipase A1, phosphospholipase A2, or a phospholipase B is used as a phospholipase in the method. More preferably the phospholipase used in the method is an isolated polypeptide having phospholipase activity according to the invention, in particular a phospholipase of the invention obtained from *Fusarium oxysporum*.

For further details reference is made to a working example herein (vide infra).

The invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

MATERIALS AND METHODS

Deposited organisms:

Fusarium oxysporum DSM No. 2672 comprises the phospholipase encoding DNA sequence of the invention.

Escherichia coli DSM 11299 containing the plasmid comprising the full length cDNA sequence, coding for the phospholipase of the invention, in the shuttle vector pYES 2.0.

Other strains:

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (MATa; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; cir+).

E. coli strain: DH10B (Life Technologies)

Plasmids:

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (Invitrogen)

pA2PH10 (See example 1)

General molecular biology methods

Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990).

Enzymes for DNA manipulations were used according to the specifications of the suppliers.

Enzymes for DNA manipulations

Unless otherwise mentioned all enzymes for DNA manipulations, such as *e.g.* restriction endonucleases, ligases etc., are obtained from New England Biolabs, Inc.

Phospholipase Activity Assay based on NEFA-C test:

Substrate: L-α-lysophosphatidylcholine (Sigma).

Substrate: Soybean Lecithin (Sigma #P3644). Used to measure phospholipase A activity.

Nefa-C test kit is from Wako Chemicals Germany.

Buffer: 20 mM NaOAc pH 4.5

Substrate solution: 10 mg substrate in 1 mL milli Q water and 1 mL buffer (make enough substrate solution to all samples)

1. 15 μl enzyme is added to 150 μl substrate solution
2. Incubation for 10 min. at 40°C
3. 30 μl is transferred to 300 μl reagent 1 (from Nefa kit)
4. Incubation for 10 min. at 37°C
5. Addition of 600 μl reagent 2 (from Nefa-kit)
6. Incubation for 10 min. at 37°C
7. Absorption of final reaction product is measured at 550 nm, according to Nefa-kit instructions.

The enzyme activity that produces 1 μmol of fatty acid per minute of the enzyme reaction was defined as 1 unit.

Expression cloning in yeast

Expression cloning in yeast was done as comprehensively described by H. Dalboege et al. (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953), which are hereby incorporated as reference.

All individual steps of Extraction of total RNA, cDNA synthesis, Mung bean nuclease treatment, Blunt-ending with T4 DNA polymerase, and Construction of libraries was done according to the references mentioned above.

Fermentation procedure of *Fusarium oxysporum* DSM No. 2672 for mRNA isolation:

Fusarium oxysporum DSM No. 2672 was cultivated in YPD medium for 4 days at 30C. 10 µl Supernatant was tested for phospholipase activity in the plate assay described below.

mRNA was isolated from mycelium from this culture as described in (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953).

Identification of positive yeast clones (plate assay):

Identification of positive yeast clones (i.e. clones which comprise a gene encoding for phospholipase activity) was done as described below.

The yeast transformants are plated on SC agar containing 2% glucose and incubated for 3 days at 30°C. A cellulose acetate filter (OE67, Schleicher & Schuell) is placed on top of the cells and then transferred to to plates containing SC agar and 2% Galactose with the cells on top of the filter. After 3 days of incubation at 30°C the filter with cells is transferred to substrate plates. Positive clones are identified as colonies giving rise to a blue-green zone in the substrate plate under the colony.

The substrate plates are made the following way: 2.5 g agar (BA-30 INA Agar®, Funakoshi Co. Ltd.) is added to 137.5 ml of H_2O, heated to boiling in a microwave oven. After cooling to about 60°C, 30 ml of the following mixture is added: 62.5 ml 0.4 M Tris-HCl buffer (pH 7.5) and 50 ml 3% Lipoid E80 (Lipoid GmbH, D-67065 Ludwigshafen, Germany) dissolved in 2% Triton X-100 (v/v) and 0.5 ml 2% Brilliant Green solution in H_2O. The concentration of the substrate is important as a too high concentration can give rise to background activity from the yeast cells.

Isolation of a cDNA gene for expression in *Aspergillus*:

A phospholipase-producing yeast colony is inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube is shaken for 2 days at 30°C. The cells are harvested by centrifugation for 10 min. at 3000 rpm.

DNA is isolated according to WO 94/14953 and dissolved in 50 ml water. The DNA is transformed into *E. coli* by standard procedures. Plasmid DNA is isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis. The cDNA insert is excised using appropriate restriction enzymes and ligated into an Aspergillus expression vector.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

Protoplasts may be prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12, which is hereby incorporated by reference.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$). Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Test of *A. oryzae* or *Aspergillus niger* transformants

Each of the *A. oryzae* transformants are inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation at 30°C, the supernatant is removed. 20 µl of supernatant is loaded into holes punched in a substrate plate (*vide supra*). After 1-24 hours, phospholipase activity appears as a blue-green zone around the hole.

Fed batch fermentation

Fed batch fermentation was performed in a medium comprising maltodextrin as a carbon source, urea as a nitrogen source and yeast extract. The fed batch fermentation was performed by inoculating a shake flask culture of *A. oryzae* host cells in question into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at pH 7.0 and 34°C the continuous supply of additional carbon and nitrogen sources were initiated. The carbon source was kept as the limiting factor and it was secured that oxygen was present in excess. The fed batch cultivation was continued for 4 days.

Isolation of the DNA sequence shown in SEQ ID No. 1

The phospholipase encoding part of the DNA sequence shown in SEQ ID No. 1 coding for the phospholipase of the invention can be obtained from the deposited organism *Escherichia coli* DSM 11299 by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

Media

YPD: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

10 x Basal salt: 75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H_2O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

SC-agar: SC-URA, 20 g/l agar added.

SC-variant agar: 20 g agar, 20 ml 10 x Basal salt, H_2O ad 900 ml, autoclaved

PEG 4000 (polyethylene glycol, molecular weight = 4,000) (BDH, England)

EXAMPLES

EXAMPLE 1

Fermentation of *Fusarium oxysporum* phospholipase

A culture of *Fusarium oxysporum*, DSM 2672 on an agar slant was transferred to five 500 ml shaking flasks, each with 100 ml of Bouillon-3 medium, and shaken at 30°C for 1 day (200 rpm, amplitude 2,5 cm).

The composition of Bouillon-3 medium was as follows:

Peptone	6 g/l
Trypsin digested casein	4 g/l
Yeast extract	3 g/l
Meat extract	1.5 g/l
Glucose	1 g/l

The medium was autoclaved at 121°C for 40 minutes.

The culture broth of these Bullion-3 shake flasks was used as a seed culture for inoculating 20 500 ml shake flasks, each with 200 ml PL-1 medium.

The composition of the PL-1 medium was as follows:

Peptone	10 g/l
Tween®-80	12 g/l
$MgSO_4;7H_2O$	2 g/l
$CaCl_2;2H_2O$	0.1 g/l
pH before autoclaving	6.0

The medium was autoclaved at 121°C for 40 minutes.

Each PL-1 shake flask was inoculated with 0.5-2 ml of Bullion-3 culture broth, and shaken with 200 rpm (amplitude 2.5 cm) at 30°C for 5 days. The culture broth from the shake flasks was pooled at harvest, totaling 3.9 l with an enzyme yield of 53 LU/ml.

EXAMPLE 2

Purification of Phospholipase

Step 1) One liter Fermentation supernatant was centrifuged and precipitate was discarded. Supernatant was then adjusted to 0.8 M ammonium acetate by adding solid ammonium acetate.

Step 2 :- Hydrophobic chromatography :-Toyopearl butyl 650 C matrix was purchased from Toso Hass (Rohm and Haas company Germany). Fifty ml column was packed with the matrix. The column was washed with 50 % ethanol and subsequently with water. Column was then equilibrated with 0.8 M ammonium acetate. Fermentation supernatant adjusted with 0.8 M ammonium acetate was then applied on the column. Unbound material was then washed with 0.8 M ammonium acetate until all the UV absorbing material (280 nm) was removed.

Column was then eluted with water and subsequently with 50% ethanol.

Phospholipase activity was determined at pH 4.5 and 40°C using NEFA kit as described in above. Fractions containing activity in water and alcohol eluate were pooled. Activity were assayed at pH 4.5 using NEFA kit assay.

Fractions containing phospholipase activity were then pooled and dialyzed and concentrated using Amicon ultrafiltration membrane with cut of 10 kDa.

Step 3:-Negative absorption on DEAE fast flow chromatography.

DEAE FF was bought from Pharmacia and 50 ml column was packed with the matrix.

Column was then washed as described by the manufacturer and equilibrated with 25 mM Tris acetate buffer pH 7.

The dialysed and concentrated sample was then adjusted to pH 7 and conductance to 2 mSi. and applied on anionic exchanger DEAE FF column.

Activity was collected as effluent. The activity does not bind to anion exchanger at pH 7.

Effluent from DEAE FF containing activity was concentrated and dialyzed using Amicon membrane with cut of 10 kDa. and buffer 25 mM Sodium acetate buffer pH 6.

Gelfiltration on Superdex 75.

Superdex 75 prepacked column Hiload Tm 16/60 from Pharmacia was washed and equilibrated with 25 mM Sodium acetate pH 6 containing 150 mM NaCl.

Two ml of the concentrated effluent from anion exchanger containing phospholipase activity at pH 4.5 and 40 degrees was applied on the superdex column.

The activity was separated by gel filtration with a flow rate of 1 ml /minute.

EXAMPLE 3

Characterization of purified phospholipase obtained from *Fusarium oxysporum*

The molecular weight of the phospholipase enzyme was determined using 4 to 20 % SDS-PAGE precasted plates from Novex Tm. Molecular weight of the protein was determined under reducing condition as described before.

For the *F. oxysporum* phospholipase the molecular weight was found to be 29-30 kDa under reducing conditions.

The isoelectric point was determined by using Ampholine PAGE plates from Pharmacia.

For the *F. oxysporum* pI of the protein was found to be around neutral pH, preferably in the range 5.8 to 6.8.

Thermostability of phospholipase

Thermostability of phospholipase from *Fusarium oxysporum* was investigated by means of DSC (Differential Scanning Calorimetry). The thermal denaturation temperature, Td, was taken as the top of denaturation peak in thermograms (Cp vs. T) obtained after heating of enzyme solutions at a constant programmed heating rate.

Experimental:

A DSC II from Hart Scientific (Utah, US, 1993) was used for the experiments.

50 mM buffered solutions were used as solvent for the enzyme (approx. 2 mg/ml) at either pH 10 (50 mM Glycine buffer), pH 7 (50 mM HEPES buffer+10 mM EDTA) or pH 4 (50 mM Citrate buffer). Enzyme was purified according to example 2 above.

750 µl enzyme solution was transferred into standard 1 ml sealable hastelloy ampoules from Hart Scientific. Ampoules were loaded into the calorimeter and cooled to 5°C for 15 min. thermal equilibration prior to the DSC scan. The DSC scan was performed from 5°C to 95°C at a scan rate of approx. 90 K/hr.. Denaturation temperatures were determined at an accuracy of approx. +/- 2°C.

Results:

Table No 1: Top to denaturation peak as a function of pH

pH	Td(°C)
4	57°C
7	62°C
10	55°C

Note that these experiments were performed in the absence of an oil matrix that might influence enzyme stability significantly. The DSC results indicate a maximal stability near neutral pH.

Assuming irreversible thermal denaturation, an relevant performance temperature in an industrial application such as degumming of oils (US 5.264.367) are at least approx. 10 degrees lower than the Td-temperatures listed in table No 1 above.

Aminoterminal sequence

Aminoterminal analysis was determined by using Edman degradation with Applied Biosystem equipment (ABI 473A protein sequencer, Applied Biosytem, USA) carried out as described by manufacturer.

N-terminal sequence(s):

For the *F. oxysporum* phospholipase the N-terminal sequence is:

N-terminal A-V-G-V-T-T-T-D-F-S-N-F-K-F-Y-I

The N-terminal amino acid "A" (Ala) is position 31 in SEQ ID NO 2. This indicate the mature phospholipase enzyme of the invention starts at position 31 in SEQ ID No 2.

Consequently the mature sequence is from 31-346 in SEQ ID no 2.

EXAMPLE 4

Phospholipase A activity

The phospholipase A activity was determined with Soybean Lecithin as substrate as described above (NEFA test bases assay) at pH 4.5 at 40°C.

The F. oxysporum phospholipase showed significant phospholipase A activity at the conditions described above.

EXAMPLE 5

Activity toward L-α-lysophosphatidylcholine

The phospholipase activity was determined with L-α-lysophosphatidylcholine as substrate as described above (NEFA test bases assay) at pH 4.5 at 40°C.

The *F. oxysporum* phospholipase showed significant activity against L-α-lysophosphatidylcholine at the conditions described above.

EXAMPLE 6

Phospholipase activity in monolayer setup

A monolayer equipment (zero-order trough, KSV5000, KSV Instruments, Finland) has been used to evaluate the activity of various enzymes toward the phospholipid DDPC (Di Dicanoyl (C10) Phosphatidyl Choline).

Experiments

On a thoroughly purified surface of a buffer solution (10 mM TRIS, pH 8.0, 25° C) a monolayer of DDPC was spread from a chloroform solution. After relaxation of the monolayer (evaporation of chlorofom) the surface pressure is adjusted to 15 mN/m, corresponding to a mean molecular area of DDPC of approx. 63 Å²/molec. An buffer solution (see above) containing approximately 60 μg (micro gram) enzyme is injected through the monolayer into the subphase of the reaction compartment (cylinder with area 1520 mm^2 and volume 30400 mm^3) in the "zero-order trough". Enzymatic activity is manifested through the speed of a mobile barrier compressing the monolayer in order to maintain constant surface pressure as insoluble substrate molecules are hydrolysed into more water soluble reaction products. Having verified that the aqueous solubility of the reaction products (capric acid and DPC) are considerable higher than for DDPC the number of DDPC-molecules hydrolyzed pr. minute by the enzyme is estimated from the mean molecular area (MMA) of DDPC.

Results

Table 2. Activity of enzymes towards DDPC in a monolayer setup.

Enzyme	Activity (nmol/min) *)
Sigma P9279 (PLA2 from bee venom, 850 U/mg)	1.9
Enzyme from *Fusarium oxysporum*	2.7
Candida antarctica B component lipase	0
Candida antarctica A component lipase	0
Recombinant Guinea Pig pancreatic Lipase (rGPL)	0.2

*) Calculated from reduction in monolayer area pr. unit time induced by the presence of enzyme.

"Enzyme from *F. oxysporum*" in table 2 is a phospholipase of the invention, purified as described in Example 2.

Conclusion

No phospholipase activity was detected for most of the enzymes except for lipases obtained Guinea pig lipase, which showed minor phospholipase activity.

The phospholipase of the invention obtained from *Fusarium oxysporum* showed surprisingly high significant phospholipase activity.

Consequently, in the present invention the term "phospholipase activity", used herein in connection with a phospholipase of the invention, is defined as an activity which in the "monolayer phospholipase assay" shown above is at least 0.25 nmol/min, Enzyme dose: 60 μg; more preferably at least 0.40 nmol/min, Enzyme dose: 60 μg; more preferably at least 0.75 nmol/min, Enzyme dose: 60 μg; more preferably at least 1.0 nmol/min, Enzyme dose: 60 μg; more preferably at least 1.25 nmol/min, Enzyme dose: 60 μg; and even more preferably at least 1.5 nmol/min, Enzyme dose: 60 μg.

The term "lipase with phospholipase side activity" is accordingly defined as a lipase with a phospholipase side activity wherein the phospholipase side activity in the "monolayer phospholipase assay" shown in Example 6 is less than the above mentioned figures specifying phospholipase activity.

Example of a lipase with phospholipase side activity according to the definitions herein are the Guinea pig lipase shown in table 2 above. This is having a phospholipase side activity in the "monolayer phospholipase assay" which is less than 0.25 nmol/min, Enzyme dose: 60 μg.

EXAMPLE 7

Cloning and expression of a phospholipase from *Fusarium oxysporum* DSM No. 2672

Cloning and expression was done by using the Expression cloning in yeast technique as described above.

mRNA was isolated from *Fusarium oxysporum*, DSM No. 2672, grown as described above with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *Fusarium oxysporum*, DSM No. 2672, consisting of approx. $9x10^5$ individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Phospholipase-positive colonies were identified and isolated on substrate plates (*vide supra*). cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above. The DNA sequence of the cDNA encoding the phospholipase is shown in SEQ ID No. 1 and the corresponding amino acid sequence is shown in SEQ ID No. 2. In SEQ ID No. 1 DNA nucleotides from No 23 to No. 1060 define the phospholipase encoding region. The part of the DNA sequence in SEQ ID NO 1, which is encoding the mature part of the phospholipase is position 113 to 1060, which correspond to amino acid position 31-346 in SEQ ID NO 2.

The cDNA is obtainable from the plasmid in DSM 11299.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the phospholipase in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the phospholipase gene was purified. The gene was subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmid pA2PH10.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

Test of *A. oryzae* transformants

Each of the transformants were tested for enzyme activity as described above. Some of the transformants had phospholipase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the phospholipase in *Aspergillus oryzae*.

EXAMPLE 8

Degumming of crude edible oil

Equipment for carrying out enzymatic degumming

The equipment consists of a 1 l jacketed steel reactor fitted with a steel lid, a propeller (600 rpm), baffles, a temperature sensor, an inlet tube at the top, a reflux condenser (4 °C) at the top, and an outlet tube at the bottom. The reactor jacket is connected to a thermostat bath. The outlet tube is connected via silicone tubing to a Silverson in-line mixer head equipped with a "square hole high shear screen", driven by a Silverson L4RT high shear lab mixer (8500 rpm, flow ca. 1.1 l/minute).The mixer head is fitted with a cooling coil (5-10 °C) and an outlet tube, which is connected to the inlet tube of the reactor via silicone tubing. A temperature sensor is inserted in the silicone tubing just after the mixer head. The only connection from the reactor/mixer head system to the atmosphere is through the reflux condenser.

General procedure for carrying out enzymatic degumming

All cooling and thermostat equipment is turned on. Then 0.6 l (ca. 560 g) of oil is loaded in the reactor, which is kept at about the temperature needed for the specific experiment. The lab mixer is turned on, whereby the oil starts to circulate from the reactor to the mixer head and back to the reactor. The system is allowed to equilibrate for about 10 minutes, in which period temperature is fine tuned. The pre-treatment period starts with addition of 0.6 g (2.86 mmol) of citric acid monohydrate in 27 g of MilliQ water (added water vs. oil equals 4.8% w/w; [citric acid] in water phase = 106 mM, in water/oil emulsion = 4.6 mM), which sets t = 0. At t = 30 minutes a suitable amount of 4 M NaOH

solution is added. At t = 35 minutes samples are drawn for P-analysis and pH determination. Just after this the required amount of enzyme solution is added (end of pre-treatment period). Samples for P-analysis and pH determination are drawn at t = 1, 2, 3.5, 5, 6 hours, and then the reaction is stopped.

Phosphorus analysis:

Sampling for P-analysis:

Take 10 ml of water in oil emulsion in a glass centrifuge tube. Heat the emulsion in a boiling water bath for 30 minutes. Centrifuge at 5000 rpm for 10 minutes. Transfer about 8 ml of upper (oil) phase to a 12 ml polystyrene tube, and leave it (to settle) for 12-24 hours. After settling draw about 1-2 g from upper clear phase for P-analysis.

P-analysis was carried out according to procedure 2.421 in "Standard Methods for the Analysis of Oils, Fats, and Derivatives, 7.th ed. (1987)":

Weigh 100 mg of MgO (leicht, Merck #5862) in a porcelain dish and heat with a gas burner. Add 1-2 g of oil and ignite with a gas burner to afford a black, hard mass. Heat in a Vecstar furnace at 850 °C for 2 hours to afford a white ash. Dissolve the ash in 5 ml of 6 M HNO_3, and add 20 ml of reagent mix. Leave for 20 minutes. Measure absorbance at 460 nm (use a blank (5 ml HNO_3 + 20 ml reagent mix) for zero adjustment). Calculate by using calibration curve.

pH determination

Take 2 ml of water in oil emulsion and mix with 2 ml of MilliQ water. After phase separation, pipette off top oil layer. Measure pH in aqueous phase with pH electrode Orion. Measurements are transformed to "real" pH values by formula

$$pH_{real} = pH_{measured} - 0.38.$$

(A calibration curve was obtained by dissolving 0.6 g of citric acid monohydrate in 27 g of DI water; pH of this solution was measured by pH electrode Orion (pH_{real}). 100 µl were mixed with 2 ml MilliQ water and pH of this solution was measured by pH electrode Orion ($pH_{measured}$). pH of the citric acid solution was changed gradually by adding NaOH solution, and for each adjustment dilution and pH measurements were carried out as described above.)

Examples

Enzymatic degumming of crude rapeseed oil (phosphorus content 609 ppm).

Experiments A and B were carried out according to "General procedure for carrying out enzymatic degumming" above.

Experiment A (reference)

0.6 l (580 g) of crude rape seed oil is loaded in the equipment and heated at 60 °C. At t = 30 min. 1.43 ml (5.7 mmoles) of 4 M NaOH solution is added, which yields a pH of about 5.6. At t = 35 min. 30 μl (300 Unit) of Lecitase 10L (obtained from Novo Nordisk A/S) is added. The measured phosphorus content in the oil phase after centrifuging as well as the pH values in the aqueous phase are given in Table 3.

Table 3. Results from degumming of crude rapeseed oil with Lecitase.

Time (hours)	Phosphorus content in oil phase	pH
0	609	
0.58	146	5.6
1.0	127	5.6
2.0	88	5.7
3.5	61	5.7
5.0	44	5.6
6.0	34	5.8

Experiment B

0.6 l (581 g) of crude rape seed oil is loaded in the equipment and heated at 40 °C. At t = 30 min. 1.07 ml (4.3 mmoles) of 4 M NaOH solution is added, which yields a pH of about 5.4. At t = 35 min. 1 ml of a purified solution (example 2) of phospholipase from *F. oxysporum* is added. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase are given in Table 4.

Table 4. Results from degumming of crude rapeseed oil with phospholipase from *F. oxysporum*.

Time (hours)	Phosphorus content in oil phase	pH
0	609	
0.58	149	5.4
1.0	91	5.3
2.0	13	5.4
3.5	11	5.3
5.0	13	5.4
6.0	10	5.2

SEQUENCE LISTING

SEQ ID No. 1 shows a cloned DNA sequence of the invention, comprising a DNA sequence encoding an enzyme exhibiting phospholipase activity.

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1170 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Fusarium oxysporum
(B) STRAIN: DSM 2672

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:23..1063

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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TTGGAGAATA TTCCTTGTCA CG ATG CTT CTT CTA CCA CTC CTC TCG GCC ATC        52
                         Met Leu Leu Leu Pro Leu Leu Ser Ala Ile
                           1               5                  10

ACC CTC GCG GTA GCC AGT CCT GTA GCT CTC GAC GAC TAC GTC AAC TCT       100
Thr Leu Ala Val Ala Ser Pro Val Ala Leu Asp Asp Tyr Val Asn Ser
                    15                  20                  25

CTT GAG GAG CGA GCT GTT GGT GTC ACT ACA ACC GAC TTC AGC AAC TTC       148
Leu Glu Glu Arg Ala Val Gly Val Thr Thr Thr Asp Phe Ser Asn Phe
                30                  35                  40

AAG TTC TAC ATC CAA CAC GGC GCC GCA GCT TAC TGC AAC TCT GAA GCC       196
Lys Phe Tyr Ile Gln His Gly Ala Ala Ala Tyr Cys Asn Ser Glu Ala
            45                  50                  55

GCA GCT GGT TCC AAG ATC ACC TGC TCC AAC AAT GGC TGT CCA ACC GTT       244
Ala Ala Gly Ser Lys Ile Thr Cys Ser Asn Asn Gly Cys Pro Thr Val
        60                  65                  70

CAG GGC AAC GGA GCG ACC ATC GTG ACA TCT TTC GTT GGC TCC AAG ACA       292
Gln Gly Asn Gly Ala Thr Ile Val Thr Ser Phe Val Gly Ser Lys Thr
    75                  80                  85                  90

GGT ATC GGT GGC TAC GTC GCG ACA GAC TCT GCC CGA AAG GAA ATC GTC       340
Gly Ile Gly Gly Tyr Val Ala Thr Asp Ser Ala Arg Lys Glu Ile Val
                95                  100                 105

GTC TCG TTC CGC GGA AGC ATC AAT ATT CGA AAC TGG CTT ACC AAC CTC       388
Val Ser Phe Arg Gly Ser Ile Asn Ile Arg Asn Trp Leu Thr Asn Leu
            110                 115                 120

GAC TTC GGC CAG GAA GAC TGC AGT CTC GTC TCT GGA TGC GGT GTG CAC       436
Asp Phe Gly Gln Glu Asp Cys Ser Leu Val Ser Gly Cys Gly Val His
        125                 130                 135
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TCT GGC TTC CAG CGA GCC TGG AAT GAG ATC TCG TCT CAA GCA ACC GCT        484
Ser Gly Phe Gln Arg Ala Trp Asn Glu Ile Ser Ser Gln Ala Thr Ala
    140                 145                 150

GCT GTT GCC TCC GCC CGC AAG GCG AAC CCT TCT TTC AAC GTC ATT TCT        532
Ala Val Ala Ser Ala Arg Lys Ala Asn Pro Ser Phe Asn Val Ile Ser
155                 160                 165                 170

ACA GGC CAC TCC CTT GGA GGT GCC GTG GCC GTT CTT GCT GCC GCA AAC        580
Thr Gly His Ser Leu Gly Gly Ala Val Ala Val Leu Ala Ala Ala Asn
                175                 180                 185

TTG AGA GTC GGT GGA ACA CCC GTC GAT ATT TAC ACC TAC GGC TCT CCC        628
Leu Arg Val Gly Gly Thr Pro Val Asp Ile Tyr Thr Tyr Gly Ser Pro
            190                 195                 200

CGT GTC GGA AAC GCG CAG CTC TCA GCC TTC GTC TCA AAC CAG GCT GGT        676
Arg Val Gly Asn Ala Gln Leu Ser Ala Phe Val Ser Asn Gln Ala Gly
        205                 210                 215

GGA GAG TAC CGC GTT ACA CAC GCT GAT GAC CCT GTC CCC CGT CTC CCT        724
Gly Glu Tyr Arg Val Thr His Ala Asp Asp Pro Val Pro Arg Leu Pro
    220                 225                 230

CCT CTG ATC TTC GGA TAC AGG CAC ACA ACT CCT GAG TTC TGG CTG TCC        772
Pro Leu Ile Phe Gly Tyr Arg His Thr Thr Pro Glu Phe Trp Leu Ser
235                 240                 245                 250

GGC GGT GGA GGC GAC AAG GTT GAC TAC ACC ATC AGC GAT GTC AAG GTC        820
Gly Gly Gly Gly Asp Lys Val Asp Tyr Thr Ile Ser Asp Val Lys Val
                255                 260                 265

TGT GAG GGT GCT GCC AAC CTT GGA TGC AAC GGT GGA ACT CTT GGT TTG        868
Cys Glu Gly Ala Ala Asn Leu Gly Cys Asn Gly Gly Thr Leu Gly Leu
            270                 275                 280

GAT ATT GCT GCT CAT CTG CAT TAC TTC CAG GCG ACT GAC GCC TGT AAC        916
Asp Ile Ala Ala His Leu His Tyr Phe Gln Ala Thr Asp Ala Cys Asn
        285                 290                 295

GCT GGT GGC TTC TCT TGG CGA CGA TAC AGA AGC GCC GAG AGC GTC GAC        964
Ala Gly Gly Phe Ser Trp Arg Arg Tyr Arg Ser Ala Glu Ser Val Asp
    300                 305                 310

AAG AGG GCC ACC ATG ACT GAT GCC GAG CTT GAG AAG AAG CTG AAC TCT       1012
Lys Arg Ala Thr Met Thr Asp Ala Glu Leu Glu Lys Lys Leu Asn Ser
315                 320                 325                 330

TAT GTC CAG ATG GAT AAG GAG TAT GTG AAG AAT AAC CAG GCC CGC TCT       1060
Tyr Val Gln Met Asp Lys Glu Tyr Val Lys Asn Asn Gln Ala Arg Ser
                335                 340                 345

TAA CGAGGGTATG AGGTTTGATG GGAAATGACA TGATTCATGA ACGAAACCAT            1113
 *

AGTACATATG ATGCAAATAG GATATAAAAA CATATTTCAT TCACTAGCTT TACACAA        1170
```

SEQ ID No. 2 shows the amino acid sequence of a phospholipase of the invention

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 346 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) *MOLECULE TYPE: protein*

(xi) *SEQUENCE DESCRIPTION: SEQ ID NO: 2:*

```
Met Leu Leu Leu Pro Leu Leu Ser Ala Ile Thr Leu Ala Val Ala Ser
 1               5                  10                  15

Pro Val Ala Leu Asp Asp Tyr Val Asn Ser Leu Glu Glu Arg Ala Val
            20                  25                  30

Gly Val Thr Thr Thr Asp Phe Ser Asn Phe Lys Phe Tyr Ile Gln His
        35                  40                  45

Gly Ala Ala Ala Tyr Cys Asn Ser Glu Ala Ala Ala Gly Ser Lys Ile
    50                  55                  60

Thr Cys Ser Asn Asn Gly Cys Pro Thr Val Gln Gly Asn Gly Ala Thr
65                  70                  75                  80

Ile Val Thr Ser Phe Val Gly Ser Lys Thr Gly Ile Gly Gly Tyr Val
                85                  90                  95

Ala Thr Asp Ser Ala Arg Lys Glu Ile Val Val Ser Phe Arg Gly Ser
            100                 105                 110

Ile Asn Ile Arg Asn Trp Leu Thr Asn Leu Asp Phe Gly Gln Glu Asp
        115                 120                 125

Cys Ser Leu Val Ser Gly Cys Gly Val His Ser Gly Phe Gln Arg Ala
    130                 135                 140

Trp Asn Glu Ile Ser Ser Gln Ala Thr Ala Ala Val Ala Ser Ala Arg
145                 150                 155                 160

Lys Ala Asn Pro Ser Phe Asn Val Ile Ser Thr Gly His Ser Leu Gly
                165                 170                 175

Gly Ala Val Ala Val Leu Ala Ala Ala Asn Leu Arg Val Gly Gly Thr
            180                 185                 190

Pro Val Asp Ile Tyr Thr Tyr Gly Ser Pro Arg Val Gly Asn Ala Gln
        195                 200                 205

Leu Ser Ala Phe Val Ser Asn Gln Ala Gly Gly Glu Tyr Arg Val Thr
    210                 215                 220

His Ala Asp Asp Pro Val Pro Arg Leu Pro Pro Leu Ile Phe Gly Tyr
225                 230                 235                 240

Arg His Thr Thr Pro Glu Phe Trp Leu Ser Gly Gly Gly Gly Asp Lys
                245                 250                 255

Val Asp Tyr Thr Ile Ser Asp Val Lys Val Cys Glu Gly Ala Ala Asn
            260                 265                 270

Leu Gly Cys Asn Gly Gly Thr Leu Gly Leu Asp Ile Ala Ala His Leu
        275                 280                 285

His Tyr Phe Gln Ala Thr Asp Ala Cys Asn Ala Gly Gly Phe Ser Trp
    290                 295                 300

Arg Arg Tyr Arg Ser Ala Glu Ser Val Asp Lys Arg Ala Thr Met Thr
305                 310                 315                 320
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Asp Ala Glu Leu Glu Lys Lys Leu Asn Ser Tyr Val Gln Met Asp Lys
                  325                 330                 335

Glu Tyr Val Lys Asn Asn Gln Ala Arg Ser  *
            340                 345
```

CLAIMS

1. A cloned DNA sequence encoding a polypeptide having phospholipase A activity wherein the DNA sequence is obtained from a filamentous fungus.

2. A cloned DNA sequence encoding an enzyme exhibiting phospholipase A and/or phospholipase B activity, which DNA sequence is selected from the group comprising of:

(a) the phospholipase A encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Escherichia coli* DSM 11299;

(b) the DNA sequence shown in positions 23-1063 in SEQ ID NO 1 or its complementary strand;

(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 70% homologous with said DNA sequence;

(d) a DNA sequence which hybridizes with the DNA sequence shown in positions 23-1063 in SEQ ID NO 1 at low stringency;

(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequences of (b) or (d), but which codes for a polypeptide having exactly the same amino acid sequences as the polypeptide encoded by these DNA sequences; and

(f) a DNA sequence which is a allelic form or fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

3. An isolated polypeptide having phopholipase A and/or phospholipase B activity which is obtained from a filamenteus fungus and comprising the amino acid sequence "YRSAESVDKRAT", or the amino acid sequence defined in (a) where one or two of the amino acid sequences have been modified.

4. A cloned DNA sequence encoding an enzyme exhibiting phopholipase A and/or phospholipase B activity, which DNA sequence comprises

(a) the DNA sequence "TAC AGA AGC GCC GAG AGC GTC GAC AAG AGG GCC ACC" (positions 941-976 in SEQ ID NO 1); or

(b) an analogue of the DNA sequence defined in (a) which hybridizes with the DNA sequence shown in positions 941-976 in SEQ ID NO 1 at low stringency.

5. An isolated polypeptide having phopholipase A activity which is obtained from a strain of the genus *Fusarium* and has

(a) PLA activity in the pH range 3-7, measured at 40°C;

(b) a molecular mass of 29 ± 10 kDa, as determined by SDS-PAGE;

(c) an isoelectric point (pI) in the range 4.5-8; and/or

(d) a thermal denaturation temperature (Td) in the range between 52°C to 65°C, measured at pH 4.

6. The isolated polypeptide according to claim 5, wherein the *Fusarium* strain is *Fusarium oxysporum*, in particular the *Fusarium oxysporum* DSM No. 2672.

7. The cloned sequence according to claim 2 or 4, wherein the DNA sequence is obtained from a microorganism, preferably a filamentous fungus, a yeast, or a bacteria.

8. The cloned sequence according to any of claims 1, 2, 4 or 7 wherein the phospholipase A is a phospholipase A1; and/or the isolated polypeptide according to claims 3, 5, or 6 wherein the phospholipase A is a phospholipase A1.

9. The cloned sequence according to any of claims 1, 2, 4 or 6 wherein the phospholipase A is a phospholipase A2; and/or the isolated polypeptide according to claims 3, 5 or 6, wherein the phospholipase A is a phospholipase A2.

10. The cloned DNA sequence according to any of claims 7-9 in which is the DNA sequence is obtained from a strain of the class *Pyrenomycetes*, such as the genera *Fusarium*, in particular a strain of *Fusarium oxysporum*.

11. The cloned DNA sequence according to claim 10, in which the DNA sequence is cloned from or produced on the basis of a DNA library of the strain *Fusarium oxysporum* DSM No. 2672.

12. A recombinant expression vector comprising a cloned DNA sequence according to any of claims 1, 2, 4, and 7-11.

13. A host cell comprising a cloned DNA sequence according to any of claims 1, 2, 4, and 7-11 or a recombinant expression vector according to claim 12.

14. The host cell according to claim 13, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

15. The host cell according to claim 14, which is a strain of *Aspergillus*, in particular a strain of *Aspergillus niger* or *Aspergillus oryzae*.

16. The host cell according to claim 14, which is a strain of the genus *Fusarium*.

17. The host cell according to claim 16, being the strain *Fusarium oxysporum* DSM No. 651.96.

18. A method of producing an enzyme exhibiting phospholipase activity, the method comprising culturing a host cell according to any of claims 13-17 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

19. An isolated enzyme exhibiting phospholipase activity, characterized in (i) being free from homologous impurities and (ii) said enzyme is produced by the method according to claim 18.

20. An isolated enzyme exhibiting phospholipase A and/or B activity selected from the group comprising of:

(a) a polypeptide encoded by the phospholipase A and/or B enzyme encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in Escherichia coli DSM 11299;

(b) a polypeptide having an amino acid sequence as shown in positions 31-346 of SEQ ID NO 2;

(c) an analogue of the polypeptide defined in (a) or (b) which is at least 70 % homologous with said polypeptide; and

(d) an allelic form or fragment of (a), (b) or (c).

21. Use of the phospholipase according to any of claims 3, 5-6, and 19-20 in a process comprising treatment of a phospholipid or lysophospholipid with the phospholipase so as to hydrolyze fatty acyl groups.

22. Use according to the preceding claim wherein the phospholipid or lysophospholipid comprises lecithin or lysolecithin.

23. Use according to claim 21 or 22 wherein the treatment is conducted at pH 1.5-5 (preferably 2-4) and 30-70°C.

24. Use according to any of claims 21-23 in a process for improving the filterability of an aqueous solution or slurry of carbohydrate origin which contains phospholipid.

25. Use according to the preceding claim wherein the solution or slurry contains a starch hydrolysate, particularly a wheat starch hydrolysate.

26. Use according to any of claims 21-23 in a process for making bread, comprising adding the phospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread.

27. Use according to any of claims 21-23 in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the phospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

28. An isolated substantially pure biological culture of the deposited strain *Escherichia coli* DSM No. 11299.

29. A method for reducing the content of phosphorus containing components in a crude edible oil or a semicrude oil, which method comprises contacting said oil at a pH from 3-6 with an aqueous solution of a phospholipase which is emulsified in the oil until the

phosphorus content of the oil is reduced to less than 11 ppm, and then separating the aqueous phase from the treated oil.

30. The method according to claim 29, wherein the phospholipase is a phospholipase A_1, phospholipase A_2, or a phospholipase B.

31. The method according to claim 30, wherein the phospholipase is a phospholipase according to any of claims 3,5,6,8,9,19, and 20.

ABSTRACT

The present invention relates to an enzyme with phospholipase activity, a cloned DNA sequence encoding the enzyme with phospholipase activity, a method of producing the enzyme, and the use of said enzyme and enzyme composition for a number of industrial applications.

PTO/SB/58 (07-03)
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Application Number	Filed
60/039791	03-04-97

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I hereby request access under 37 CFR 1.14(a)(1)(iv) to the application file record of the above-identified ABANDONED application, which is identified in, or to which a benefit is claimed, in the following document (as shown in the attachment):

United States Patent Application Publication No. _____, page, _____ line _____.

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US006103505A

United States Patent [19]

Clausen et al.

[11] Patent Number: 6,103,505

[45] Date of Patent: *Aug. 15, 2000

[54] METHOD FOR REDUCING PHOSPHORUS CONTENT OF EDIBLE OILS

[75] Inventors: Ib Groth Clausen, Hillerød; Shamkant Anant Patkar, Lyngby; Kim Borch, Copenhagen K, all of Denmark; Martin Barfoed, Raleigh, N.C.; Kim Clausen, Tølløse, Denmark; Claus Crone Fuglsang, Niva, Denmark; Lone Dybdal, Copenhagen Ø, Denmark; Torben Halkier, Birkerød, Denmark

[73] Assignee: Novo Nordisk A/S, Bagsværd, Denmark

[*] Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

[21] Appl. No.: 08/988,111

[22] Filed: Dec. 9, 1997

Related U.S. Application Data

[63] Continuation of application No. 60/039,791, Mar. 4, 1997.

[30] Foreign Application Priority Data

Dec. 9, 1996	[DK]	Denmark	1408/96
Dec. 16, 1996	[DK]	Denmark	1432/96
Feb. 21, 1997	[DK]	Denmark	0190/97
Feb. 26, 1997	[DK]	Denmark	0211/97
Nov. 11, 1997	[DK]	Denmark	1283/97

[51] Int. Cl.[7] C12P 7/64; C12N 9/20; C07K 1/00; C07H 21/04

[52] U.S. Cl. 435/134; 435/198; 435/252.3; 435/320.1; 536/23.2; 536/23.7; 530/350

[58] Field of Search 435/134, 198, 435/252.3, 320.1, 69.1; 536/23.2, 23.7; 530/350

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Primary Examiner—Ponnathapura Achutamurthy
Assistant Examiner—Tekchand Saidha
Attorney, Agent, or Firm—Steve T. Zelson, Esq.; Reza Green, Esq.

[57] ABSTRACT

The present invention relates to a method for reducing the content of phosphorous containing components in an edible oil comprising a high amount of non-hydratable phosphorous content, wherein the method comprises use of a phospholipase. Further the present invention relates to an enzyme with phospholipase activity, a cloned DNA sequence encoding the enzyme with phospholipase activity, a method of producing the enzyme, and the use of enzyme for a number of industrial applications.

4 Claims, 2 Drawing Sheets

NO DISK NEEDED

POSITION		ID NO.	DATE
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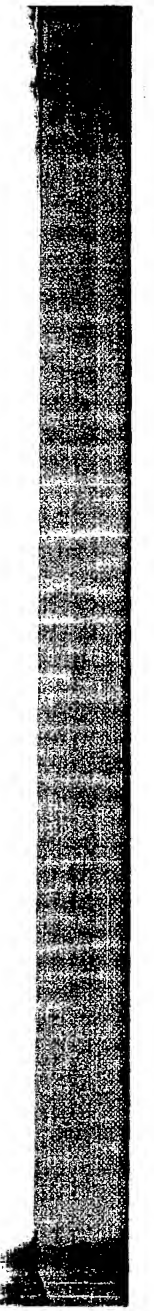
VERIFIER			
CORPS CORR.			
SPEC. HAND			
FILE MAINT			
DRAFTING			



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PATENT APPLICATION

60039791

APPROVED FOR LICENSE

INITIALS APR 04 1997

Date Entered or Counted

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Date Received or Mailed

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